UCRL-JC-125921
PREPRINT

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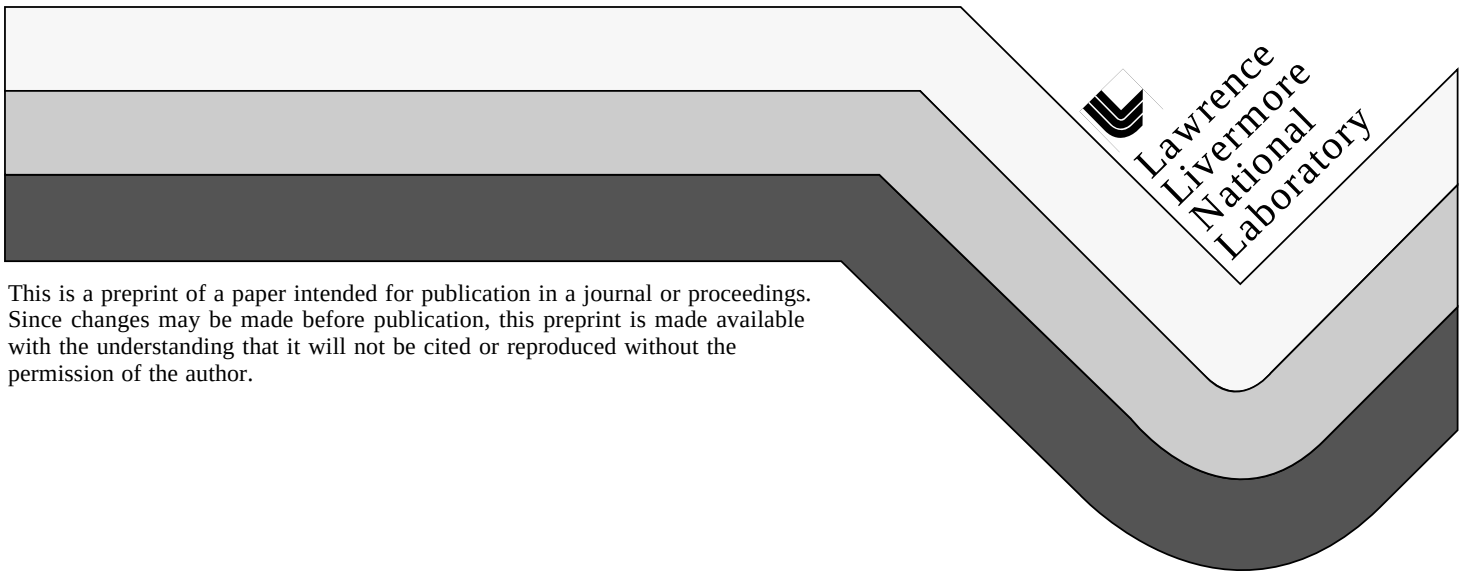
This paper was prepared for submittal to the
***Scientific Conference on Chemical & Biological Defense Research***
***Aberdeen Proving Ground, MD***
***November 19-20, 1996***

**November 1996**



# ATTOMOLE DETECTION OF ISOTOPE-LABELED COMPOUNDS IN CHEMICAL DEFENSE RESEARCH

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ABSTRACT.

AMS detects $^{14}C$ at zeptomole to femtomole sensitivities. We detected the effect of ChE-blocking pyridostigmine bromide on the CNS uptake of a pyrethroid insecticide at scaled human-equivalent exposures in rats. Significant blood to brain protection from permethrin dosed at 5 μg/kg is seen in the CNS of rats receiving pyridostigmine bromide pretreatments in chow at 2mg/kg/day. The synergy of these compounds was suggested as a precursor to some symptoms of "Gulf War Syndrome".

Quantitative studies of effects of chemical nerve agents [1], organophosphate (OP) insecticides [2], or non-OP insecticides [3], and the amelioration of these effects by cholinesterase (ChE) inhibitors [4], often rely on the statistical analyses of qualitative endpoints from a large number (> 100) of dosed animals. The median lethal dose (LD50) is quantitated for dose response with a statistically significant number of animals at each dose level. Toxic effects may also be quantitated as the minimal dose producing an observable result, such as muscle twitching, using enough individual animals for statistical significance. These studies find lethal toxicity or indicate onset of prophylactic treatment, and these outcomes and the quantization are relevant. However, non-lethal exposures to toxic compounds are more frequent in almost all human occupations: industrial, agricultural, or military. Low exposures result from small leaks of containment vessels, distance from a point of high concentration release, or indirect contaminants left on surfaces, within streams, or in plant material. Outcomes from these sublethal exposures may not be predicted by the high dose studies of qualitative endpoints.

Incipient harm from low dose exposures is evaluated by tissue doses in critical target organs. Tissue doses are often determined by HPLC, MS, or IA identification and quantification of the metabolized compounds in dissected organs. These techniques depend on prior identification of all chemical species and can exclude unexpected metabolites. Radioisotopic labels in an administered chemical quantify all resultant species of the compound, but the sensitivity required

for detecting low concentrations in tissues required high specific activities available from short-lived isotopes or multi-labeled molecules. These high specific activities lead to laboratory hazards, hazardous waste, or mixed toxic/radioactive waste. We developed technology, Accelerator MS (AMS), to quantitate radioisotope-labeled compounds to attomole levels containing femtoCuries of long-lived isotopes such as $^{14}C$. This technology is better known as a standard method for radioisotope geochronology [5]. We have applied AMS to pharmacokinetic studies of carcinogens found in foods [6 7] and of environmental toxins [8] at very low doses relevant to normal human exposures.

We used AMS in a preliminary study of the synergistic effects of a non-OP insecticide, permethrin (PER), and a nerve agent pretreatment, pyridostigmine bromide (PYR), in rats at doses equivalent to those purportedly received by soldiers operating under threat of nerve agent attack in uniforms impregnated with the insecticide. Synergism between these chemicals was argued to be a contributor to the "Gulf War Syndrome" [9, 10, 11, 12]. Abou-Donia, et al. [13] demonstrated a synergy in adult hens at relatively high doses of permethrin (500 mg/kg/day) .We report here a preliminary experiment in rats exposed to permethrin at 4.8 μg/kg/day with and without previous chronic exposure to pyridostigmine bromide at 7.9 mg/kg/day. Snodgrass, et al. [14] predict a permethrin exposure of 0.6 μg/kg/day from treated uniforms; and the established pretreatment dose of pyridostigmine bromide is 1.29 mg/kg/day [15]. Our rat doses represent human equivalent doses of 0.7 μg/kg of permethrin and 2.0 mg/kg/day assuming an intraspecies scaling factor of 7 for hydrophobic compounds and 4 for hydrophilic compounds.

## Materials and Methods

### *Chemicals*

[$^{14}C$, RL]3- (2,2dichloroethenyl) -2,2-dimethylcyclopropanecarboxilic acid (3phenoxyphenyl) methyl ester, Z9999: permethrin, and 3-dimethylamino-carbonyloxy-N-methylpyridium bromide, P9797: pyridostigmine bromide, were obtained from Sigma Chemical Co. (St. Louis, MO) . Permethrin was labeled at 9.8 mCi/mmol with an average of 15.7% of the molecules containing a $^{14}C$ and was diluted in ethanol to 50 μCi/ml. All other reagents were analytical grade and obtained from common commercial sources. Permethrin, 1.59μg/ml, was added to a dosing solution of 40% glycerol, 10% ethanol, and 50% acetate buffer solution (0.9% NaCl by weight and 0.012% glacial acetic acid by volume) .

### *Animals*

Adult male Sprague-Dawley rats, from Simonsen Labs (Gilroy, CA) , were housed two to a cage in standard laboratory conditions. Rat chow was obtained from Purina Mills Inc, Certified Laboratory Chow # 5002C. A portion of rat chow was ground and impregnated with PYR at 104 mg/kg of feed and re-pelletized. Feed and water were provided ad libitum.

### *Protocol*

Eight rats were fed a normal diet prior to dosing with permethrin. Four rats maintained in the same environment were provided chow containing PYR. Equilibrium was assured by maintenance on this chow for 10 days. The total ingested dose was obtained by weighing the presented and remaining food. Rats ranged in weight from 275-305 g after the 10 day pretreatment period and ingested an average of 7.75 mg/kg/day, equivalent to a human dose of 1.94 mg/kg/day. Eight rats (4 on PYR chow, 4 on normal chow) were dosed IP with 3 ml/kg $^{14}C$-permethrin dosing solution (4.75 μg/kg) , a human equivalent dose of 0.68 μg/kg. Four rats on plain chow were undosed controls for measurement of laboratory contamination. Two rats from each group (control, plain chow, pretreated chow) were sacrificed one hour after dosing, with the remainder of

the rats sacrificed at 24 hours after dosing. Urine and feces were collected from metabolism cages containing a control and a 24 hr. PER dosed animal on normal chow.

*Samples*

After $CO_2$ asphyxiation, circulating blood was sampled by cardiac puncture and frozen. The brains, upper spinal columns, livers, spleens, and femoral nerves were removed and placed on ice for later sampling. Carcasses were frozen for later sampling. Feces and urine were separately homogenized and counted for $^{14}C$ using liquid scintillation. Approximately 5 mg samples were taken from the frontal lobe and the cerebellum of the brains and the spinal columns. The samples were rinsed but not perfused to remove blood. Both coagulate and liquid were sampled from the thawed blood. Samples were fully combusted and reduced to graphite [16] for measurement by AMS. A more detailed description of the methods involved in making AMS measurements of biological samples is provided in Creek, et al. [17] and Vogel, et al. [16].

*Statistics*

Values are expressed as x ± SE. Statistical analysis for the difference in mean values was done with Student's t test using Data Desk analysis software (Data Description, Ithaca, NY) .

Results

Table 1 summarizes the tissue concentration of PER-equivalents in pg/g. All eight rats received 4.8 μg/kg [$^{14}C$]PER. The rats indicated in the Table received a 10 day chronic dose of 2 mg/kg/day PYR. The 5 μg/kg administered [$^{14}C$]PER dose resulted in 2-5 μg/kg (2000 - 5000 pg/g) blood levels one hour after exposure, falling to 0.3 μg/kg levels within 24 hours. PER equivalents found in the cerebellum, frontal lobe and spinal column reached 0.5 μg/kg one hour after dosing and fell to 0.03 μg/kg within 24 hours. The chronic pretreatment of the rat hosts over 10 days with 2 mg/kg/day doses of PYR significantly reduced the amount of PER reaching the nervous tissues by 30%, although no significant change was due to PYR in the blood levels at either the one hour or twenty-four hour points. No significant effect of PYR pretreatment is detected in the brain at twenty-four hours, but the protection of the spinal cord is significant at twenty four hours.

Urines collected from a PER dosed animal in a metabolism cage show a 6% excretion in the first 3 hours and a total excretion of 47% over 24 hours, with only 2.5% of that in the feces. This crude data implies a biological half life of 15 hours for this initially metabolized pool. Very long-term metabolism studies are possible using the sensitivity of AMS, but this study halted at 24 hours. The data in Table 1 imply crude, 2-point lifetime calculations, giving shorter half-lifes of 5.6 ± 0.9 hours for components in the CNS and a more uncertain 7.1 ± 2.0 hours in the blood components.

**Table 1**

**[$^{14}$C]Permethrin Tissue Concenration[1] (pg/g)**

| | | Brain[2] ± (n) | | Spinal ± (n) | | Plasma[3] ± (n) | | Solids[4] ± (n) | |
|---|---|---|---|---|---|---|---|---|---|
| **1 Hour** | | | | | | | | | |
| **Rat** | | | | | | | | | |
| | | 621 | 51 (4) | 577 | 99 (3) | 3500 | 190 (2) | 18000 | 5400 (2) |
| **2** | | 688 | 51 (3) | 424.6 | 3.2 (3) | 2260 | 100 (2) | 4370 | 770 (3) |
| | *Ave* | ***650*** | ***36 (7)*** | ***501*** | ***56 (6)*** | *2880* | *370 (4)* | *9800* | *3800 (5)* |
| **3** | PYR | 561 | 49 (4) | 426 | 33 (3) | 3000 | 130 (2) | 6740 | 170 (3) |
| **4** | PYR | 351 | 12 (4) | 257 | 12 (3) | 890 | 15 (2) | 1530 | 140 (2) |
| | *Ave* | ***456*** | ***46 (8)*** | ***342*** | ***41 (6)*** | *1940* | *610 (4)* | *4700* | *1300 (5)* |
| **24 Hou** | | | | | | | | | |
| **Rat** | | 15.26 | 0.49 (4) | 45 | 18 (3) | 262.2 | 2.1 (2) | 235 | 57 (3) |
| **6** | | 37.3 | 6.6 (4) | 34.2 | 7.0 (3) | 560 | - (1) | 362 | 13 (3) |
| | *Ave* | *26.3* | *5.2 (8)* | ***39.6*** | ***8.9 (6)*** | *360* | *100 (3)* | *299* | *39 (6)* |
| **7** | PYR | 510 | 22 (4) | 17.2 | 1.1 (3) | 356 | 13 (2) | 581 | 33 (3) |
| **8** | PYR | 23.82 | 0.61 (4) | 3.10 | 0.44 (2) | 338 | 38 (2) | 357 | 22 (3) |
| | *Ave* | 38 | *11 (8)* | ***11.6*** | ***3.5 (5)*** | *347* | *17 (4)* | *469* | *53 (6)* |

1 Datasets in bold type show an effect of treatment by pyridostigmine bromide at a 95% confidence level (2 sample t-interval).

2 Cerebellum and frontal lobe.

3 Liquid from thawed blood.

4 Solids from thawed blood.

Discussion

AMS tissue dose measurements are independent of the sample size presented, since the isotope ratio is measured. Natural $^{14}$C occurs in the biosphere at 1.2 ppq and is subtracted from the measured ratio. $^{14}$C concentrations of equivalent dissected tissues from control animals were subtracted from the measured isotope ratios before correction of the excess isotope concentration to PER equivalents. This integrates all $^{14}$C contamination arising in the animal handling, the dissection, and the tissue sampling. While the one hour control rats had $^{14}$C concentrations consistent with contemporary living animals, the twenty four hour controls were 10-20% higher in $^{14}$C than expected, indicating an incorporation of $^{14}$C from their environment over that period.

The sensitivity of AMS is demonstrated by the 3.10 ± 0.44 pg/g measure of the PER equivalents in the spinal nerve tissue of rat 8, a part per quadrillion (ppq) measurement. The precision of AMS is demonstrated by a standard error of 2 amol on the 15 amol measured. Larger

standard errors of other measurements represent the difficulty of contamination-free dissection and sample definition at attomole sensitivities. Small amounts of blood having 100 times the amount of PER as the nerve tissues are likely responsible for the variations. Laboratory furniture, equipment, and tools are also sources of large uncertainties in AMS measurements [17].

The difference in the estimated biological half-life through external and internal measures implies a two-component system, as found by Anadón, et al. [18]. The longer externally derived time reflects a storage in body lipids. Our blood data half-lives are similar to the 8.7 and 12.3 hour blood elimination times found by Anadón, et al. for their intravenous dose of 46 mg/kg and oral dose of 460 mg/kg respectively. The equivalence in blood clearance over a dose difference of 5 orders of magnitude implies a simple excretion metabolism that is not enzyme limited. These authors find an accumulation in nervous tissue with hydrophobic PER several times higher in the cerebellum than the acid and alcohol metabolites. This trend is reversed in the plasma, and there is a higher concentration in the nervous tissue overall than in plasma. We measure only PER-equivalents as $^{14}C$ with no metabolite discrimination, but we see a factor of 10-50 less PER-equivalents in the CNS than in blood. Although the excretion follows a similar pattern, the tissue penetration and distribution is very different at very high exposures and at our dose. This may reflect an altered balance of PER and its metabolites throughout the animal at this lower dose.

It was expected, and shown by Abou Donia, et al [13] in chickens, that PYR blocks PER metabolism by hepatic and erythocyte esterases, leading to longer bodily retention of the hydrophobic PER. Metabolism and elimination in nerves is also slowed by the inhibition of nerve esterases. Our data support a very different view, with no significant additional retention of the PER due to PYR pretreatment in the blood components, nor a significant additional component of PER in the CNS at twenty four hours. A lower amount of PER or its metabolites is able to cross the blood brain barrier. If the metabolites of PER are restricted in crossing the blood-brain barrier by their polarity, then their presence in the brain tissue is due to metabolism in the CNS and/or a transport of one or both primary metabolites across this barrier. Assuming that the hydrophobic PER in our lower dose enters nerve tissue equally well as at high doses, our data are consistent with a blockage of a barrier transport protein by PYR that is required by one of the metabolites. Given multiple pathways for metabolites of PER to enter the CNS, the 30% reduction of PER-equivalents caused by PYR is reasonable and consistent in magnitude with reductions of organophosphates seen in rat brains after physostigmine pretreatment [19].

We conclude that PYR does not cause an increase of PER in the nervous system of mammals. Our present data are insufficient to confirm our speculations about cause. However, AMS is an excellent tool for further work. We chose the cerebellum, frontal lobe, and top of the spinal column as our test tissues for ease in initial dissection. These parts are less perfused by plasma-borne chemicals than the hypothalamus and amygdala, more likely sites of damage giving rise to symptoms described as “Gulf War Syndrome”. AMS sensitivity is needed in studies of such localized nerve tissue.

We are developing rapid separation protocols to detect the presence and amount of receptor protein binding. We can use AMS to test hypotheses about the existence of transport proteins that are affected by PYR. The same protocols can be used with $^{14}C$-labeled pyridostigmine to quantify the binding of PYR to brain esterase, as well as hepatic and erythrocytic esterase. Alternatively, the transport protein hypothesis can be tested using young rats before the blood brain barrier is intact. We should see no protection from PYR in that case.

## CONCLUSIONS

Fate and distribution of chemical compounds is determined in biological and environmental systems at realistic exposure levels using AMS, eliminating the uncertain extrapolation from high doses, and revealing natural metabolic pathways without inducing unnatural expressions of enzymes, cell death, or gross chemical change. AMS detection of radioisotopic labels in compounds administered to laboratory hosts or to human volunteers permits toxicological and pharmaceutical research at sub-lethal, and even sub-toxic levels. Sensitivities at ppq are achieved. Administered doses are nCi to pCi of $^{14}C$. Wastes, used vessels, and instruments are disposed as non-radioactive. The work areas are not radioactive material handling areas. AMS is now only available in a few institutions, but newer, more inexpensive, and smaller spectrometers will soon be available. The Center for AMS at LLNL is the present source of most biological AMS measurements in the world.

We have shown that pyridostigmine bromide decreases the amount of permethrin and/or its metabolites reaching the central nervous systems of mammals when doses equivalent to those used in nerve agent pretreatment are administered. In a similar manner, AMS could be used to determine the gross, tissue specific, and molecular dependent distribution, metabolism, elimination and effects of chemical agents and the drugs or pretreatments to combat them.

## ACKNOWLEDGMENTS

Work was performed under the auspices of the U.S. Department of Energy at Lawrence Livermore National Laboratory under contract W-7405-Eng-48. Support was provided by the University of California CLC Program and by a fellowship from the National Physical Science Consortium to NHP. Initiation and useful discussions with Bruce Hammock, UC Davis are acknowledged, as are useful discussions with Mary-Lou Oster-Granite of UC Riverside.

## REFERENCES

1. Koplovitz, I., Gresham, V.C., Dochterman, L.W., Kaminskis, A. & Stewart, J.R. *Arch Toxicol* **66**, 622-8 (1992).
2. Ozkutlu, U., Long, J.P., Cannon, J.G., Sahin, M.F. & Liang, C. *Arch Int Pharmacodyn Ther* **329**, 331-42 (1995).
3. Cantalamessa, F. *Arch Toxicol* **67**, 510-3 (1993).
4. Maxwell, D.M., Brecht, K.M., Doctor, B.P. & Wolfe, A.D. *J Pharmacol Exp Ther* **264**, 1085-9 (1993).
5. Vogel, J.S., Turteltaub, K.W., Finkel, R. & Nelson, D.E. *Analytical Chemistry* **67**, A353-A359 (1995).
6. Turteltaub, K.W., Vogel, J.S., Frantz, C.E. & Shen, N. *Cancer Research* **52**, 4682-4687 (1992).
7. Frantz, C.E., *et al. Carcinogenesis* **16**, 367-373 (1995).
8. Bogen, K.T., Keating, G.A. & Vogel, J.S. *Prediction of Percutaneous Penetration* **4B**, 195-198 (1996).
9. Medicine, I.o. *Health Consequence of Service During the Persian Gulf War: Initial Findings and Reccommendations of Immediate Action* (National Academy Press, Washington, D.C., 1995).
10. Gavaghan, H. *Nature* **369**, 8 (1994).

11. *Environ Health Perspect* **103**, 792-3 (1995).
12. Pennisi, E. *Science* **272**, 479-80 (1996).
13. Abou-Donia, M.B., Wilmarth, K.R., Jensen, K.F., Oehme, F.W. & Kurt, T.L. *J Toxicol Environ Health* **48**, 35-56 (1996).
14. Snodgrass, H.L. *J Toxicol Environ Health* **35**, 91-105 (1992).
15. Dunn, M.C. & Sidell, F.R. *Journal of the American Medical Association* **262**, 649 - 652 (1989).
16. Vogel, J.S. & Turteltaub, K.W. *Trac-Trends In Analytical Chemistry* **11**, 142-149 (1992).
17. Creek, M.R., *et al. Nuclear Instruments & Methods In Physics Research Section B-Beam Interactions With Materials and Atoms* **92**, 454-458 (1994).
18. Anadón, A., Martinez-Larrañaga, M.R., Diaz, M.J. & Bringas, P. *Toxicology and Applied Pharmacology* **110**, 1-8 (1991).
19. Desphande, S.S., Viana, G.B., Kauffman, F.C., Rickett, D.L. & Albuquerque, E.X. *Fundamental and Applied Toxicology* **6**, 566-577 (1986).

Table 1 [14C]Permethrin Tissue Concenration (pg/g)1

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| 1 Hour | | | | | | | | | |
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| | Ave | 650 | 36 (7) | 501 | 56 (6) | 2880 | 370 (4) | 9800 | 3800 (5) |
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| 4 | PYR | 351 | 12 (4) | 257 | 12 (3) | 890 | 15 (2) | 1530 | 140 (2) |
| | Ave | 456 | 46 (8) | 342 | 41 (6) | 1940 | 610 (4) | 4700 | 1300 (5) |
| 24 Hour | | | | | | | | | |
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| | Ave | 3811 (8) | | 11.6 | 3.5 (5) | 347 | 17 (4) | 469 | 53 (6 |

Datasets in bold type show an effect of treatment by pyridostigmine bro
t-interval). Cerebellum and frontal lobe.
Liquid from thawed blood.
Solids from thawed blood.